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14. ABSTRACT

Farnesyltransferase inhibitors (FTIs) block the post-translational processing of signaling proteins, such as Ras, that have key roles in breast cancer biology. In phase II trials, FTIs have exhibited clinical benefit toward a subset of breast cancer patients. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. To overcome these hurdles, mechanisms determining whether breast cancer tumors are FTI-sensitive or -resistant in vivo must be understood. Accordingly, this recently funded project is developing molecular imaging strategies that for the first time specifically detect the ability of FTIs to inhibit farnesylation in tumors of living animals. Our initial proposed imaging strategy uses chimeric transcription factors fused to the prenylation domains H-Ras or Cdc42, which localize to the nucleus upon inhibition of prenylation. When unprenylated, the fusion chimeras bind their cognate promoter, driving expression of firefly luciferase, a reporter that can be readily imaged in cells and animals with an ultrasensitive, cooled CCD camera. This strategy should offer the opportunity to visualize over time the action of FTIs and GGTIs toward specific, biologically relevant prenylation-dependent proteins in tumors of living animals.

15. SUBJECT TERMS
Breast cancer, farnesyltransferase inhibitors, drug resistance, molecular imaging

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Introduction

Prenyltransferase inhibitors (FTIs & GGTIs) block post-translational processing of Ras-like GTPases that have key roles in breast cancer. In phase II trials, FTIs have exhibited clinical benefit toward a subset of breast cancer patients. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. GGTIs are also being developed for cancer therapy. To overcome these hurdles, mechanisms determining whether breast cancer tumors are sensitive or resistant to FTIs or GGTIs in vivo must be understood. These mechanisms remain elusive because signaling networks regulating proliferation, survival and migration of breast cancer cells in vitro versus in vivo can be strikingly different, and because conventional biochemical means of detecting FTI or GGTI action in tumors are insensitive, invasive, or correlate poorly with FTI or GGTI action. Therefore, novel means of elucidating mechanisms of FTI or GGTI activity in tumors are required. Accordingly, this project will develop molecular imaging strategies that for the first time specifically detect the ability of FTIs or GGTIs to inhibit prenylation in tumors of living animals.

Body

The main objective is to identify mechanisms determining whether breast cancer tumors in living animals are sensitive or insensitive to FTIs or GGTIs. We will develop novel imaging technology to detect the action of a clinically employed FTIs or GGTIs in human breast cancer cell lines in vitro and in mouse xenograft models of human breast cancer. With this system, the specific aims of the project are:

Aim 1--Determine whether FTIs or GGTIs inhibit prenylation in tumors.

Aim 2--Determine whether prenylation blockade occurs in FTI- or GGTI-sensitive vs. -insensitive tumors.

Aim 3--Determine whether FTase or GGTase knock down or Erk, Akt or mTOR inhibition augments FTI or GGTI sensitivity in vitro or in vivo.

Aim 1: Determine whether FTIs inhibit farnesylation in tumors

The majority of our effort in the first 12 months of funding has been directed toward developing, validating and calibrating the desired bioluminescence reporter. Our strategy to image FTI or GGTI action in vivo is shown in **Figure 1**, which summarizes the final strategy we have employed. Our initial proposed imaging strategy used a plasmid that constitutively expressed a chimeric transcription factor comprised of the DNA binding domain (DBD) of the yeast Gal4 transcription factor, the VP16 transcriptional activation domain (AD), a nuclear localization sequence (NLS), and H-Ras. However, repeated analysis with this construct in breast cancer cell lines yielded a high degree of variability and lack of durable signals with this construct. Thus, we sought an alternative strategy. Following an extensive series of experiments with various permutations of this basic construct, we found a chimeric transcription factor fused to the prenylation domains of the CaaX proteins H-Ras or Cdc42 which showed promise to localize to the nucleus upon inhibition of prenylation. We have now constructed plasmids

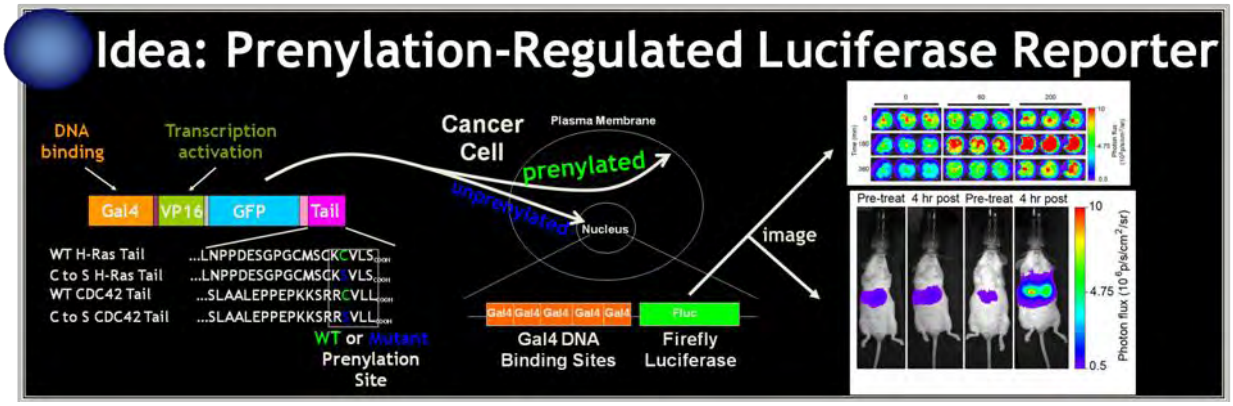
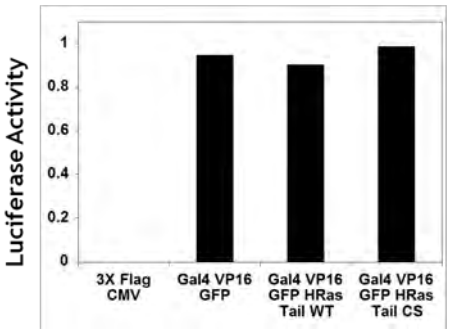


Figure 1. Concept of prenylation-regulated luciferase reporter.

that constitutively express chimeric transcription factors comprised of the DNA binding domain (DBD) of the yeast Gal4 transcription factor, the VP16 transcriptional activation domain (AD), a green fluorescent protein (GFP) scaffold and the CaaX domains of H-Ras or Cdc42. When farnesylated, the resultant Gal4-VP16-GFP-H-Ras-CaaX or Gal4-VP16-GFP-Cdc42-CaaX fusion proteins target the plasma membrane and thus, fail to activate a Gal4 promoter-driven luciferase reporter construct. However, when unfarnesylated, such as in response to FTI treatment, the fusion protein should now be imported into the nucleus and drive expression of the Gal4 promoter luciferase reporter. For further analysis via mutational inactivation of the CaaX box, we also engineered two mutant constructs comprising CaaX to SaaX tail substitutions as control positive probes of reporter function.

First, to document the cytotoxic effects of FTI's in the breast cancer cell lines of interest for our analysis, we first determined the cytotoxicity profiles of FTI-277 and GGTI-298, two prenylation inhibitors, on human MDA-MB-231 and MCF-7 breast cancer cells. Both compounds showed concentration-dependent cytotoxicity in both cell lines. The LD_{50} values were 20 μM and 0.01 μM for FTI-277, and 5 μM and 0.8 μM for GGTI-298, in MDA-MB-231 and MCF-7 cells, respectively. Thus, MCF-7 cells were more sensitive to both compounds under cell culture conditions.



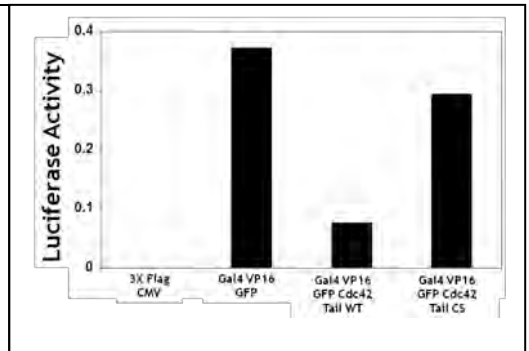


Figure 2 (left) and Figure 3 (right). Functional responsiveness of the Gal4-VP16-GFP-H-Ras-CaaX (Figure 2) and Gal4-VP16-GFP-Cdc42-CaaX (Figure 3) fusion transactivators in live cells.

Next, expression and function of the Gal4-VP16-GFP-H-Ras-CaaX and Gal4-VP16-GFP-Cdc42-CaaX chimeras were tested in MDA-MB-231 cells. As shown (**Figure 2**), the Gal4-VP16-GFP-H-Ras-CaaX was not sensitive to the

C-S mutation in the prenylation tail. In contrast, the wild-type Gal4-VP16-GFP-Cdc42-CaaX fusion showed low reporter signal under basal conditions and high signal with the C-S mutation, conforming to expectations for a prenylation responsive reporter (**Figure 3**).

To test the effect of a prenylation inhibitor on the responsiveness of the reporter, we transiently expressed the wild-type Gal4-VP16-GFP-Cdc42-CaaX fusion in MDA-MB-231 reporter cells. Increasing concentrations of GGTI-298 produced graded increases in bioluminescence output of the luciferase reporter as desired (**Figures 4 and 5**). The EC_{50} was estimated as ~7 μM, correlating well with the LD_{50} value for compound-induced cytotoxicity. Importantly, there was no effect of the compound on the Gal4-VP16-GFP fusion control or the C-S tail mutant.

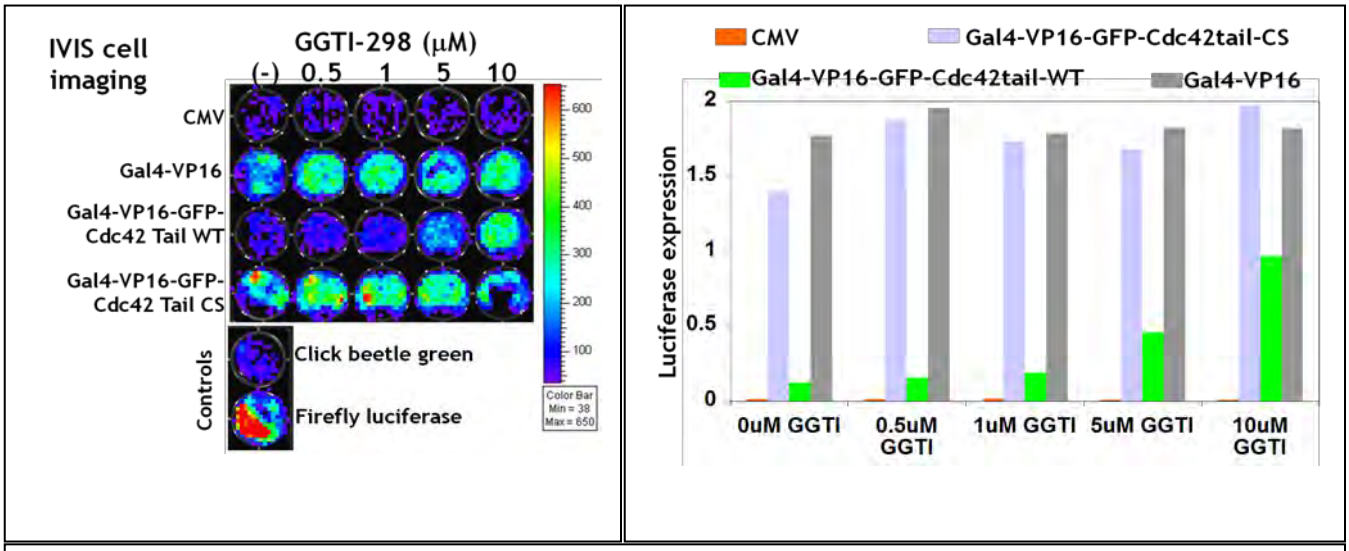


Figure 4 (left) and Figure 5 (right). Concentration-response of GGTI-mediated inhibition of prenylation in live MDA-MB-231 cells assessed with a Gal4-VP16-GFP-Cdc42-CaaX fusion transactivator of a Gal4-responsive luciferase reporter. Figure 4 shows live cell bioluminescence images and Figure 5 shows quantitative analysis of the photon flux.

Aims 2 and 3 will be pursued as planned in year 2. This novel imaging strategy will be coupled with molecular screening methods to identify mechanisms determining FTI or GGTI sensitivity and resistance in tumors. The system now will be used to compare FTI and GGTI action toward tumor xenografts derived from breast cancer cell lines that are sensitive versus resistant to FTIs or GGTIs in vitro. This strategy should offer the opportunity to visualize over time the action of FTIs and GGTIs toward specific, biologically relevant prenylation-dependent proteins in tumors of living animals. We hope to address whether FTIs or GGTIs exert their adjuvant chemotherapeutic action by inhibiting prenylation of specific, biologically relevant targets such as Cdc42, and over the long-term, to define the mechanisms responsible for FTI or GGTI sensitivity and insensitivity in vivo.

Key Research Accomplishments

- A novel prenylation responsive bioluminescence reporter system has been constructed and validated in human breast cancer cell lines.
- The imaging reporter responds in cell culture to a GGTI in a concentration-dependent manner.
- This system should provide a gain-of-function bioluminescence assay for imaging FTI action in vitro and in vivo.

Reportable Outcomes

Abstract presentation, Era of Hope Annual Meeting, 2008, Baltimore, MD:

1) Penly, A., Pichler-Wallace , A., Blumer, K., Piwnica-Worms , D. Elucidating mechanisms of prenyltransferase inhibitor action and resistance in breast cancer by bioluminescence imaging. DOD BCRP Meeting Proceedings, 2008:367.

Early Morning Session, Era of Hope Annual Meeting, 2008, Baltimore, MD:

2) Piwnica-Worms, D. Dynamic molecular imaging of signal transduction pathways in vivo. DOD BCRP Program, 2008:36.

Conclusions

Prenyltransferase inhibitors (FTIs & GGTIs) block post-translational processing of Ras-like GTPases that have key roles in breast cancer. However, FTIs have yet to be used widely in breast cancer therapy because it is not yet possible to identify patients likely to be FTI-sensitive or to use combinatorial therapy to broaden the spectrum of patients that respond to FTIs. Our strategy should offer the opportunity to visualize over time the action of FTIs and GGTIs toward specific, biologically relevant prenylation-dependent proteins in tumors of living animals. We hope to address whether FTIs or GGTIs exert their adjuvant chemotherapeutic action by inhibiting prenylation of specific, biologically relevant targets such as H-Ras and Cdc42, and over the long-term, to define the mechanisms responsible for FTI or GGTI sensitivity and insensitivity in vivo.